Gene Silencing of MK2 in Hard-to-Transfect Human U937 Cells

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Genetic modifications used to answer biological questions in cultured cells are widely used in basic research. Common transfection methods are based on viral components causing cell activation or chemical modifications of small interfering RNA and cytotoxic reagents. Here, we report a rapid and efficient approach to transfect hard-to-transfect human U937 cells via the HVJ Envelope vector system, independent from special transfection media or immobilization of cells. This protocol provides a convenient means of knocking down MAPK-activated kinase 2 in hard-to-transfect cells to study inflammation, cell adhesion, and migration characteristics.

KEY WORDS: siRNA, knockdown, nonviral transfection

INTRODUCTION

In basic research, genetic modifications, such as knockdown, inhibition, and overexpression of genes, are used to investigate the role of specific molecules. To address functional biological backgrounds, cultured cells are often used as a model system. Several cells, such as immune cells, are typically grown in suspension culture. Efficient gene silencing in suspension cells by transfection may be complicated. In the majority of cases, small interfering (si)RNA-based gene silencing methods require adherent cells cultured in nonantibiotic and serum-free media. Moreover, cytotoxic reagents or viral elements, which cause cell activation, are inherent parts of commercial transfection kits. Other methods to generate genetic modifications, such as electroporation, lead to a high level of cell death and therefore, are inefficient.[1]

In this study, we demonstrate a rapid and simple approach to knockdown the MAPK-activated kinase 2 (MK2) in suspension cells. The reviewed protocol permits an efficient transfection, independent from cell immobilization and preculturing in specific media. MK2 signaling is known to be involved in various cellular processes, such as cell growth and differentiation, cell cycle and apoptosis, actin-remodeling, and inflammation.[2–4] It has become evident that MK2 is involved in several inflammatory diseases and therefore, is a possible target to modulate the immune response.[5]

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MATERIALS AND METHODS

Cell Transfection

To create a knockdown of MK2, we transfected cells using different methods: 1) standard protocols for siRNA and small hairpin (sh)RNA transfection (sc-35855 and sc-35855-SH; Santa Cruz Biotechnology, Germany); 2) transfection reagents X-tremeGENE (Roche Diagnostics, Grenzach-Wyhlen, Germany); 3) PromoFectin-siRNA (PromoKine GmbH, Heidelberg, Germany); and 4) Lipofectamine LTX kit (purchased from Invitrogen GmbH, Darmstadt, Germany). Moreover, we tested the $CaCl_2$ method to generate a reasonable knockdown of MK2 in U937 cells. Therefore, confluent cells (90%) were split 1:10, the day before transfection. The following day, the media were replaced on each flask (RPMI 1640 + 10% FBS + 0.5% pen/strep). After 6 h, cells were transfected with MK2-shRNA. HBSS buffer (18 μL 1×1.26 mM $CaCl_2$, 0.81 mM $MgSO_4$, 5.37 mM KCl, 0.44 mM KH_2PO_4, 4.17 mM $NaHCO_3$, 136.89 mM NaCl, 0.34 mM Na_2HPO_4), 10 μL (1 μg) shRNA (Santa Cruz Biotechnology), and 1.1 μL 2.5 M $CaCl_2$ were pipetted in a sterile tube (one tube for one well of a 24-well plate). The mixtures were allowed to sit for 20 min. Afterward, the transfection mix was added dropwise to the cells in 280 μL shRNA transfection media (Santa Cruz Biotechnology) in a 24-well plate. The next day, media were removed, and 500 μL normal growth media were added to each well.

siRNA Transfection via the GenomONE–Neo EX Vector Kit

Following the manufacturer's instruction to transfect suspension cells, 10 μL vector suspension was combined with 10 μL (1 μg) siRNA. By adding 2 μL Reagent B and centrifugation for 5 min (11,000 rpm; 4°C), the siRNA

was incorporated to the hemagglutinating virus of Japan (HVJ) vector. Afterward, supernatant was removed, and the pellet was resuspended in 30 μL provided buffer. Then, 5 μL Reagent C was added. Subsequently, the reaction mix was given into a 2-mL reaction tube containing 5×10^5 cell in 500 μL normal growth media. Thereafter, the mix was centrifuged for 20 min (2000 rpm; 20°C). Then, supernatant was discarded, and pellet was resuspended in 2 mL media and incubated for 48 h at 37°C and 5% CO_2 in a six-well plate.

Western Blotting

To analyze the knockdown efficiency, Western blotting was performed. Therefore, protein was isolated from transfected and nontransfected cells with 35 μL lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.4, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 0.01% NaN_3) containing 5 μL proteinase inhibitor cocktail (Roche Diagnostics). The lysis buffer was incubated for 1 h on ice. To quantify the amounts of protein, a Bradford assay using the Bradford reagent from Carl Roth GmbH (Karlsruhe, Germany) was performed. To separate protein lysates, equal amounts of protein (100 μg), supplemented with 6× loading dye (375 mM Tris, pH 6.8, 55% glycerol, 12% SDS, 500 mM β-ME, 0.002% bromphenol blue), were separated by 10% SDS-PAGE and immunoblotted with anti-MK2 antibody (Cell Signaling Technology, New England Biolabs, Frankfurt a. M., Germany) and anti-GAPDH antibody (Cell Signaling Technology, New England Biolabs), 1:1000 on nitrocellulose (GE Healthcare, Solingen, Germany). Detection of a chemiluminescence signal after ECL (Pierce Western blotting substrate, Fisher Scientific GmbH, Schwerte, Germany) incubation was performed using the PEQLAB fusion documentation system (Fusion-FX7, PEQLAB Biotechnologie GmbH, Erlangen, Germany). Intensity of bands was quantified using the BioID software from PEQLAB.

Cell Viability Assay

We analyzed the amount of cell viability after siRNA transfection by flow cytometry. Cells were stained with 7-amino-actinomycin D ready-to-use acid dye (BD Biosciences, Heidelberg, Germany) for the exclusion of nonviable cells for 10 min at 4°C. Then, cells were washed twice in FACS buffer (1× PBS containing 0.5% BSA and 0.02% NaN_3) and analyzed with the FACSCalibur flow cytometer (BD Biosciences).

RESULTS

To investigate the physiological role of MK2 in cellular processes in response to inflammatory cytokines in the human U937[6] histiocytic lymphoma suspension cell line, we transfected cells with MK2-siRNA or -shRNA. To establish a well-defined protocol, we used several commercial transfection reagents. We initially used the siRNA transfection protocol from Santa Cruz Biotechnology. To generate an efficient knockdown, we tested varying amounts of transfection reagents and siRNA and incubated the formed siRNA complex for different time periods. Additionally, we immobilized U937 cells by serum reduction overnight and transfected cells following the manufacturer's protocols. This did not create an efficient knockdown of MK2 in U937 cells. The same procedure was performed using the MK2-shRNA with the corresponding transfection reagent from Santa Cruz Biotechnology. As a result of the failed knockdown of MK2 by using the transfection reagents from Santa Cruz Biotechnology, we combined MK2-siRNA and MK2-shRNA with generally used transfection reagents from other companies. We used the X-tremeGENE transfection reagent from Roche Diagnostics to transfer the MK2-shRNA into U937 cells. Also, we tested the Lipofectamine LTX kit, purchased from Invitrogen GmbH, to transfer siRNA or shRNA. Additionally, we used the PromoFectin-siRNA cell transfection reagent from PromoKine GmbH to bring down the expression of MK2. All tested methods failed to knockdown MK2, even when we immobilized cells or modified the protocols by changing incubation times and amounts of added substances.

Knockdown of MK2 by using common transfection reagents was inefficient; hence, we investigated further transfection methods. The $CaCl_2$ method is known to be a powerful tool to transfect cells.[7] We performed one variant of this method by adding shRNA or siRNA, HBSS buffer, and $CaCl_2$ to the cells. The treatment of cells with $CaCl_2$ caused a high level of cell death and failed to knock down MK2.

The results reviewed above suggest that the U937 human histiocytic lymphoma cell line is resistent to many transfection reagents. These findings are in accordance with recent conclusions that the human U937 cells are hard to transfect cells.[7] Nevertheless, we aimed at developing a gene-silencing protocol for MK2. As an alternative method, we used biological particles as carrier to transfer DNA or siRNA directly into target cells. We incorporated the MK2-siRNA into the HVJ Envelope vector (Cosmo Bio, Tokyo, Japan), which is a system for transfection of DNA, siRNA, oligonucleotides, proteins, and antibodies. This system contains a chimeric vector combining at least two different vector systems. The HVJ Envelope vector system is a nonviral vesicle containing only the viral fusion proteins. This procedure to generate a genetic modification is independent from chemical modification of siRNA.[8] To create a knockdown of MK2, we used the protocols of the

HVJ Envelope system kit for transfection of suspension cells. We incorporated siRNA or shRNA from Santa Cruz Biotechnology into the HVJ vector, which transferred the material into the target cells. Results showed that the transfer of shRNA was not successful, whereas the transfection of siRNA reduced the expression of MK2 (Fig. 1). Quantification of bands showed that the expression of MK2 was reduced by >90% (Fig. 2). Moreover, the analysis of cell viability showed that 85% of cells survived 48 h after transfection (data not shown). To test whether this method is a general approach to generate gene silencing of MK2 in suspension cells, we transfected Jurkat cells (human T cell line)[9] following the same protocol. The analysis of the protein expression showed a 50% decrease in the expression of MK2 (data not shown).

DISCUSSION

In this study, we used different approaches to transfect human U937 suspension cells: commercial nonviral transfection reagents, which require chemical modification of siRNA, the $CaCl_2$ method, and the HVJ Envelope vector system.

Our results showed a high transfection efficiency when MK2-siRNA and the HVJ vector were combined. In all other cases, there was no decrease of MK2 detectable. The incorporation of the siRNA by a "natural" endocytotic process does not require cell damage by chemical reagents or physical treatment. It also is independent from cell immobilization and therefore, is a safe and efficient approach to transfer molecules into cells.

There are few limitations regarding the use of this method. The sialic acid receptor, which is needed to trigger binding of the vector, is almost present in all animal cell lines. siRNA transfection of U937 cells is reported to be difficult, even with liposomal reagents and electroporation.[7] The strength of the electric field and the pulse duration are important parameters for effective compound delivery, as well as for target cell viability.[10] This method has been applied successfully; however, cell viability was quite low. Low transfection efficiency has also been reported for liposome-based nonviral tools for transient transfection of cells. The liposomes are incorporated into target cells through endocytosis, which may result in degradation of the transferred DNA by lysosomes (Cosmo Bio).

In this report, we have shown that knockdown of MK2, using the HVJ vector, is a simple and rapid method to transfect suspension cells without subjecting the cells to harsh conditions. This protocol provides a convenient means of knocking down MK2 in hard-to-transfect cells and therefore, opens a wide field for the investigation of MK2 signaling in biological processes.

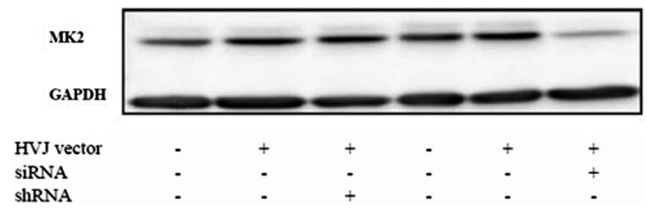

FIGURE 1

Analysis of MK2 expression. Cell lysates, generated from U937 cells transfected with MK2-shRNA (third lane) or MK2-siRNA (last lane), were separated by SDS-PAGE and immunoblotted with anti-MK2 antibody and anti-GAPDH antibody. Control lysates from native cells (first lane and fourth lane) and lysates from cells treated with the empty vector (second and fifth lanes) were used. Transfection with shRNA did not cause any difference, whereas siRNA transfection resulted in a lower level of MK2.

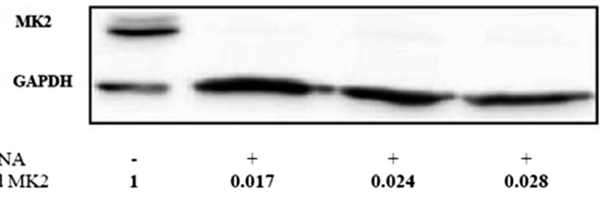

FIGURE 2

Analysis of MK2 knockdown efficiency. Cell lysates generated from native U937 cells (first lane) or U937 cells transfected with MK2-siRNA (second, third, and fourth lanes) were separated by SDS-PAGE and immunoblotted with anti-MK2 antibody and anti-GAPDH antibody. Quantification of bands from three replications of knockdown experiments were performed. MK2 was reduced by ~97%.

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